



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 9/16	A1	(11) International Publication Number: WO 94/23702
		(43) International Publication Date: 27 October 1994 (27.10.94)

(21) International Application Number: PCT/US94/04561

(22) International Filing Date: 22 April 1994 (22.04.94)

(30) Priority Data:
051,739 22 April 1993 (22.04.93) US

(71) Applicant (for all designated States except US): EMISPHERE TECHNOLOGIES, INC. [US/US]; 15 Skyline Drive, Hawthorne, NY 10532 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): MILSTEIN, Sam, J. [US/US]; 1 Knollwood Drive, Larchmont, NY 10538 (US). BARANTSEVITCH, Evgueni [RU/US]; Apartment 2B, 127 Cooper Drive, New Rochelle, NY 10801 (US).

(74) Agents: ROBINSON, Joseph, R. et al.; Darby & Darby, 805 Third Avenue, New York, NY 10022 (US).

(81) Designated States: AT, AU, BB, BG, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

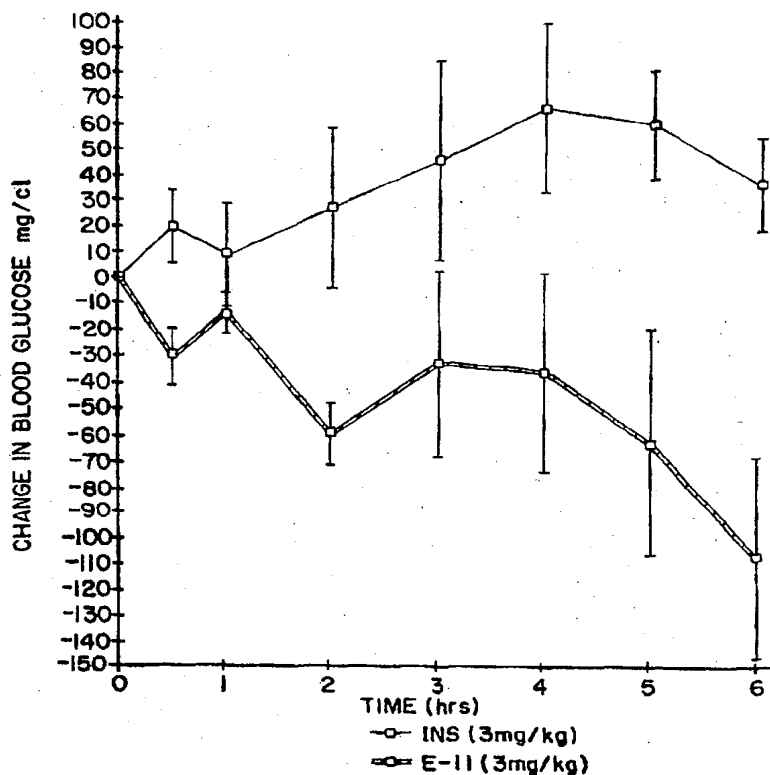
Published

With international search report.

(54) Title: MODIFIED HYDROLYZED VEGETABLE PROTEIN MICROSPHERES AND METHODS FOR PREPARATION AND USE THEREOF

(57) Abstract

Modified hydrolyzed vegetable protein microspheres and methods for their preparation and use as oral delivery systems for pharmaceutical agents are described. Figure 1 illustrates levels of glucose detected in rat serum taken from rats orally administered microsphere encapsulated insulin or raw (unencapsulated) insulin as described in example 4. Figure 2 illustrates rat serum calcium levels after oral administration of calcitonin and calcitonin encapsulated in the vegetable protein microsphere of the present invention as described in example 5. Figure 3 illustrates an HPLC trace of the hydrolyzed vegetable protein before modification. Figure 4 illustrates the change in the hydrolyzed vegetable protein after modification with benzene sulfonyl chloride in an HPLC trace.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

5

10

**MODIFIED HYDROLYZED VEGETABLE PROTEIN MICROSPHERES
AND METHODS FOR PREPARATION AND USE THEREOF**

This is a continuation-in-part of U.S. Patent application Serial No. 08/051,739, filed April 22, 1993, which is a
15 continuation-in-part of U.S. Patent application Serial No. 07/995,508, filed December 21, 1992. This invention relates to modified hydrolyzed vegetable proteins and microspheres made from them. The microspheres releasably encapsulate active agents and are suitable for oral administration to mammals. Methods for the
20 preparation of such microspheres are also disclosed.

Background of the Invention

The available means for delivering pharmaceutical and therapeutic agents to mammals often are severely limited by
25 chemical or physical barriers or both, which are imposed by the body. For example, oral delivery of many biologically-active agents would be the route of choice if not for the presence of chemical and physicochemical barriers such as extreme pH in the gut, exposure to powerful digestive enzymes, and impermeability
30 of gastrointestinal membranes to the active ingredient. Among the numerous pharmacological agents which are known to be unsuitable for oral administration are biologically active peptides and proteins, such as insulin. These agents are rapidly destroyed in the gut by acid hydrolysis and/or by proteolytic
35 enzymes.

Much research has been devoted to developing effective oral drug delivery methods and systems for these vulnerable pharmacological agents. The proposed solutions have included :

(a) co-administration of adjuvants (such as resorcinols and non-ionic surfactants polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether to increase the permeability of the intestinal walls; and

(b) co-administration of enzymatic inhibitors, such as pancreatic trypsin inhibitor, diisopropylfluorophosphate (DFF) and trasylol to avoid enzymatic degradation.

The use of such substances, in drug delivery systems, is limited however either because of their:

- (a) inherent toxicity when employed in effective amounts; or
- (b) failure to protect the active ingredient or promote its absorption; or
- (c) adverse interaction with the drug.

Liposomes as drug delivery systems have also been described. They provide a layer of lipid around the encapsulated pharmacological agent. The use of liposomes containing heparin is disclosed in U.S. Patent No. 4,239,754 and several studies have been directed to the use of liposomes containing insulin; e.g., Patel et al. (1976) FEBS Letters Vol. 62, page 60 and Hashimoto et al. (1979) Endocrinol. Japan, Vol. 26, page 337. The use of liposomes, however, is still in the development stage and there are continuing problems, including:

- (a) poor stability;
- (b) inadequate shelf life;
- (c) limited to low MW (< 30,000) cargoes;
- (d) difficulty in manufacturing;
- (e) adverse interactions with cargoes.

More recently, artificial amino acid polymers or proteinoids, forming microspheres, have been described for encapsulating pharmaceuticals. For example, U.S. Patent No. 4,925,673 (the '673 patent), the disclosure of which is hereby

incorporated by reference in its entirety, describes such microsphere constructs as well as methods for their preparation and use. The microspheres of the '673 patent are useful for encapsulating a number of active agents, however there is a need
5 in the art for microsphere carriers that can encapsulate a broader range of active agents such as lipophilic drugs.

Additionally, the method employed in the '673 patent for preparing proteinoids results in a complex mixture of high molecular weight (MW) (> 1000 daltons) and low MW (≤ 1000
10 daltons) peptide-like polymers which are difficult to separate. Moreover, small amounts of the low MW microsphere-forming proteinoids are obtained. Thus, an improved method of preparing low molecular weight sphere-forming proteinoids is also desired.

15 Summary of the Invention

The present invention relates to a modified hydrolyzed vegetable protein microsphere and to a method for preparation of such microspheres. The invention provides stable microspheres which are preparable from inexpensive hydrolyzed vegetable
20 protein, e.g. soybean protein, and a simple and economical method for making such microsphere. Microspheres made according to the invention display improved stability and performance in delivering biologically active materials to mammals.

According to the invention, modified hydrolyzed
25 vegetable microspheres are prepared by dissolving hydrolyzed vegetable protein in an aqueous alkaline solution and adding a chemical modifier which reacts with free amine residues present in the hydrolyzed protein. The pH of the reaction mixture is then lowered until the modified vegetable protein precipitates
30 out from the mixture. The recovered protein readily forms microspheres and can be used to encapsulate various cargoes such as pharmaceutical agents. The microspheres are non-toxic and can be orally or parenterally administered to mammals. Also contemplated by the present invention are dosage unit forms that
35 include these compositions.

Description of the Drawings

Figure 1 illustrates levels of glucose detected in rat serum taken from rats orally administered microsphere encapsulated insulin or raw (unencapsulated) insulin as described in Example 4.

Figure 2 illustrates rat serum calcium levels after oral administration of calcitonin and calcitonin encapsulated in the vegetable protein microsphere of the present invention as described in Example 5.

Figure 3 illustrates an HPLC trace of the hydrolyzed vegetable protein before modification.

Figure 4 illustrates the change in the hydrolyzed vegetable protein after modification with benzene sulfonyl chloride in an HPLC trace.

Detailed Description of the Invention

All patents, patent applications, and literatures cited in the specification are hereby incorporated by reference in their entirety. In the case of inconsistencies, the present disclosure, including definitions, will prevail.

The modified vegetable protein microspheres of the present invention may be prepared by reacting a hydrolyzed vegetable protein with a chemical modifying agent which reacts with free amino residues present in the protein. The modified vegetable protein is then converted into microspheres which encapsulate active ingredients, e.g. drugs. A number of advantages are obtainable by the present invention which include (a) the use of readily available and inexpensive starting materials and (b) a cost-effective method for preparing and isolating microsphere-producing modified proteins. The overall modification process is simple to perform and is amenable to industrial scale-up production.

The compositions of the subject invention are useful

for administering biologically-active agents to any animals such as birds; mammals, such as primates and particularly humans; and insects.

According to the method of the present invention, an acid or enzyme hydrolyzed vegetable protein is useful in practicing the invention. The vegetable protein generally contains titratable carboxylic acid groups (COOH) ranging between about 3 and about 8 milliequivalents/g, preferably between about 4 and about 6 milliequivalents/g, total free amino groups (NH₂) ranging between about 3 and about 9 milliequivalents/g, preferably ranging between about 4 and about 7 milliequivalents/g NH₂. The molecular weight of the vegetable protein ranges between about 100 D and about 2000 D, preferably between about 200 and about 500 D.

Hydrolyzed vegetable protein is available from a variety of commercial sources. Non-limiting examples of such sources include Ajinomoto USA, Inc. (Teaneck, NJ 07666, USA); Central Soya Co., Inc. (Fort Wayne, IN, USA); and Champlain Industries, Inc. (Clifton, NJ, USA) and additional companies listed in "Food Engineering Master", an annual publication of Chilton Co., Radnor, PA 19089, USA. A particularly preferred hydrolyzed vegetable protein in practicing this invention is available from Ajinomoto USA under the tradename AJI-EKI. This product is an acid hydrolyzed liquid soybean protein which is derived from defatted soybean meal.

If desired, a dried protein extract of the hydrolyzed vegetable protein solution may be used to prepare the modified vegetable protein of the invention. The dried protein extract is preparable by extracting the hydrolyzed vegetable solution with a suitable solvent, e.g., methanol, followed by evaporating the solvent extract.

The vegetable protein is then dissolved in aqueous alkaline solution of a metal hydroxide, e.g., sodium or potassium hydroxide, and heated at a temperature ranging between about 50°C

and about 70°C, preferably between about 50°C and about 60°C, for a period ranging between about 10 minutes and about 40 minutes, preferably about 15 minutes. The amount of alkali employed per mmole of titratable NH_2 in the vegetable protein generally ranges
5 between about 2 and about 3 mmole, preferably between about 2.2 and about 2.5 mmole. The pH of the solution generally ranges between about 8 and about 13, preferably ranging between about 9 and about 10.

Thereafter, an amine modifying agent is then added to
10 the reaction mixture while stirring. The amine modifying agents are compositions that can react with the free amino (NH_2) residues present in the protein. Some non-limiting examples of amine modifying agents useful in practicing the present invention include sulfonating agents such as benzene sulfonyl chloride and
15 acylating agents such as benzoyl chloride.

The amount of amine modifying agent in relation to the quantity of hydrolyzed vegetable protein employed is based on the equivalents of total free NH_2 in the vegetable protein. Thus, between about 0.3 and about 1.2 equivalents of modifying agent
20 are used for each molar equivalent of total NH_2 groups in vegetable protein, and preferably between about 0.6 and about 1.0 equivalents of the modifying agent for each molar equivalent of total NH_2 groups in the hydrolyzed vegetable protein.

In practicing the invention, the mixture of vegetable
25 protein and modifying agent is maintained at a temperature generally ranging between about 50°C and about 70°C, preferably between about 60°C and about 65°C for a period ranging between about 2 and about 5 hours.

The reaction is quenched by adjusting the pH of the
30 mixture with a suitable acid, e.g., concentrated hydrochloric acid, until the pH reaches between about 2 and about 3. The mixture separates on standing at room temperature to form an opaque upper layer and a dark viscous lower layer. The upper layer is discarded and modified vegetable protein is collected

from the lower layer by filtration. The crude modified vegetable protein is then dissolved in water at a pH ranging between about 9 and about 13, preferably between about 11 and about 13. Insoluble materials are removed by filtration and the filtrate is dried in vacuo. The yield of modified protein generally ranges between about 30 and about 60%, usually about 45%.

The modified vegetable protein of the present invention is soluble in alkaline aqueous solution ($\text{pH} \geq 9.0$); partially soluble in ethanol, n-butanol and 1:1 (v/v) toluene/ethanol solution and insoluble in neutral water. The titratable functional groups remaining in the vegetable protein after modification are as follows: carboxylic acid groups (COOH) ranging between about 1.5 and about 3.5 milliequivalents/g, preferably about 2.3 milliequivalents/g, amino groups (NH_2) ranging between about 0.3 and about 0.9 milliequivalents/g, preferably about 0.5 milliequivalents/g. The molecular weight of the modified vegetable protein ranges between about 200 D and about 2000 D, preferably between about 200 D and about 500 D.

The modified vegetable protein of the present invention can be used immediately to microencapsulate an active pharmacological agent or the protein can be concentrated or dried by conventional means and stored for future use.

The modified vegetable protein may be purified by fractionation on solid column supports such as alumina, using methanol/n-propanol mixtures as the mobile phase; reverse phase column supports using trifluoroacetic acid/acetonitrile mixtures as the mobile phase; and ion exchange chromatography using water as the mobile phase. When anion exchange chromatography is performed, a subsequent 0-500 mM sodium chloride gradient is employed. The modified vegetable protein may also be purified by extraction with a lower alcohol such as methanol, butanol, or isopropanol to remove low molecular weight contaminants.

The following procedure may be employed to make microspheres from purified modified vegetable protein. Modified

vegetable protein is dissolved in deionized water at a concentration ranging between about 75 and about 200 mg/ml, preferably about 100 mg/ml at a temperature between about 25°C and about 60°C, preferably about 40°C. Particulate matter remaining in the solution may be removed by conventional means such as gravity filtration over filter paper.

Thereafter, the protein solution, maintained at a temperature of about 40°C, is mixed 1:1 (V/V) with an aqueous acid solution (also at about 40°C) having an acid concentration ranging between about 0.05 N and about 2 N, preferably about 1.7 N. The resulting mixture is further incubated at 40°C for a period of time effective for microsphere formation as observed by light microscopy. In practicing this invention, the preferred order of addition is to add the protein solution to the aqueous acid solution.

Suitable acids include any acid which does not (a) adversely effect the protein, e.g., chemical decomposition; (b) interfere with microsphere formation; (c) interfere with microsphere encapsulation of cargo; and (d) adversely interact with the cargo. Preferred acids for use in this invention include acetic acid, citric acid, hydrochloric acid, phosphoric acid, malic acid and maleic acid.

In practicing the invention, a microsphere stabilizing additive preferably incorporated into the aqueous acid solution or into the protein solution, prior to the microsphere formation process. The presence of such additives promotes the stability and dispersibility of the microspheres in solution.

The additives may be employed at a concentration ranging between about 0.1 and 5 % (W/V), preferably about 0.5 % (W/V). Suitable, but non-limiting, examples of microsphere stabilizing additives include gum acacia, gelatin, polyethylene glycol, and polylysine.

Under the above conditions, the carrier forms hollow or solid matrix type microspheres wherein the cargo is distributed in a carrier matrix or capsule type microspheres encapsulating

liquid or solid cargo. If the carrier microspheres are formed in the presence of a soluble material, e.g., a pharmaceutical agent in the aforementioned aqueous acid solution, this material will be incorporated in the microspheres. In this way, one can incorporate pharmacologically active materials such as peptides, proteins, and polysaccharides as well as charged organic molecules, e.g., antimicrobial agents, which normally have poor bioavailability by the oral route. The amount of pharmaceutical agent which may be incorporated in the microsphere is dependent on a number of factors which include the concentration of agent in the microsphere forming solution, as well as the affinity of the cargo for the carrier.

Under these conditions, the modified vegetable protein molecules form hollow microspheres of less than 10 microns in diameter. If the protein microspheres are formed in the presence of a soluble material, e.g., a pharmaceutical agent in the aforementioned aqueous acid solution, this material will be encapsulated in the hollows of the microspheres and confined within the protein wall defined by the spherical structure. In this way, one can encapsulate pharmacologically active materials such as peptides, proteins, and polysaccharides as well as charged organic molecules, e.g., quinolones or antimicrobial agents, having poor bioavailability by the oral route. The amount of pharmaceutical agent which may be encapsulated by the microsphere is dependent on a number of factors which include the concentration of agent in the encapsulating solution, as well as the affinity of the cargo for the carrier.

Biologically-active agents suitable for use with carriers disclosed herein include, but are not limited to, peptides, and particularly small peptide hormones, which by themselves do not pass or only pass slowly through the gastrointestinal mucosa and/or are susceptible to chemical cleavage by acids and enzymes in the gastro-intestinal tract; polysaccharides and particularly mixtures of mucopolysaccharides; carbohydrates; lipids; or any combination thereof. Examples include, but are

not limited to, human growth hormone; bovine growth hormone; growth hormone releasing hormone; interferons; interleukin-I; insulin; heparin, and particularly low molecular weight heparin; calcitonin; erythropoietin; atrial naturetic factor; antigens; 5 monoclonal antibodies; somatostatin; adrenocorticotropin; gonadotropin releasing hormone; oxytocin; vasopressin; cromolyn sodium (sodium or disodium cromoglycate); vancomycin; desferrioxamine (DFO); or any combination thereof.

10 Additionally the carriers of the present invention can be used to deliver other active agents such as pesticides and the like.

The amount of active agent in the composition typically is a pharmacologically or biologically effective amount. However, the amount can be less than a pharmacologically or 15 biologically effective amount when the composition is used in a dosage unit form, such as a capsule, a tablet or a liquid, because the dosage unit form may contain a multiplicity of carrier/biologically-active agent compositions or may contain a divided pharmacologically or biologically effective amount. The 20 total effective amounts will be administered by cumulative units containing in total pharmacologically or biologically active amounts of biologically-active agent.

Dosage unit forms can also include any of excipients; diluents; disintegrants; lubricants; plasticizers; colorants; and 25 dosing vehicles, including, but not limited to water, 1,2-propane diol, ethanol, olive oil, or any combination thereof.

The modified vegetable protein microspheres of the invention are pharmacologically harmless and do not alter the physiological and biological properties of the active agent. 30 Furthermore, the encapsulation process does not alter the pharmacological properties of the active agent. While any pharmacological agent can be encapsulated within the protein microspheres, it is particularly valuable for delivering chemical or biological agents which otherwise would be destroyed or 35 rendered less effective by conditions encountered within the body

of the mammal to which it is administered, before the microsphere reaches its target zone (i.e., the area in which the contents of the microsphere are to be released) and which are poorly absorbed in the gastrointestinal tract.

5 The protein microspheres of the invention are particularly useful for the oral administration of certain pharmacological agents, e.g., small peptide hormones, which, by themselves, pass slowly or not at all through the gastro-intestinal mucosa and/or are susceptible to chemical cleavage by acids and enzymes
10 in the gastrointestinal tract. Non-limiting examples of such agents include human or bovine growth hormone, interferon and interleukin-II, calcitonin, atrial naturetic factor, antigens and monoclonal antibodies.

15 The particle size of the microsphere plays an important role in determining release of the active agent in the targeted area of the gastrointestinal tract. Microspheres having diameters between about ≤ 0.1 microns and about 10 microns, preferably between about 5.0 microns and about 0.1 microns, and encapsulating active agents are sufficiently small to effectively
20 release the active agent at the targeted area within the gastrointestinal tract. Small microspheres can also be administered parenterally by being suspended in an appropriate carrier fluid (e.g., isotonic saline) and injected into the circulatory system or subcutaneously. The mode of administration selected
25 will, of course, vary, depending upon the requirement of the active agent being administered. Large protein microspheres (>10 microns) tend to be less effective as oral delivery systems.

30 The size of the microspheres formed by contacting modified vegetable protein with water or an aqueous solution containing active agents can be controlled by manipulating a variety of physical or chemical parameters, such as the pH, osmolarity or ionic strength of the encapsulating solution, and by the choice of acid used in the encapsulating process.

35 The vegetable protein-derived microspheres of the present invention are suitable for oral administration of peptide

hormones, e.g., insulin, and polysaccharides, e.g., heparin, which otherwise would be quickly destroyed in the stomach. They also are suitable for protecting the stomach from gastric irritants, such as aspirin and NSAID'S. When such aspirin containing microspheres are orally administered, they pass through the gastrointestinal mucosa and release the aspirin far more rapidly than conventional enterically coated aspirin, which first must traverse the stomach and then must enter the blood-stream from the intestine after the enteric coating has dissolved.

The microspheres of the invention may be orally administered alone as solids in the form of tablets, pellets, capsules, and granulates suitable for suspension in liquids such as water or edible oils. Similarly, the microspheres can be formulated into a composition containing one or more physiologically compatible carriers or excipients, and which can be administered via the oral route. These compositions may contain conventional ingredients such as gelatin, polyvinylpyrrolidone and fillers such as starch and methyl cellulose. Alternatively, small microspheres (size less than 10 μm) can be administered via the parenteral route.

The following examples are illustrative of the invention but are not intended to limit the scope of the invention.

Example 1: **Modification of Soybean protein with benzenesulfonyl chloride**

a. Extraction of soybean protein

3.2L of acid hydrolyzed liquid soybean protein solution (AJI-EKI, Ajinomoto USA, Inc.) was reduced in vacuo to give 1440g of solid powder. This solid was extracted 3 times with methanol (2L per extraction). Methanol was removed from the pooled extracts by evaporation. The yield of soybean protein as a dark brown powder was 608 g. The functional groups of the soybean protein powder was titrated using conventional procedures. See,

for example, "A Laboratory Manual of Analytical Methods of Protein Chemistry," Vol. 1-3, Editors P. Alexander and R.J. Block, Pergamon Press, 1960 and 1961. The soybean protein contained the following functional groups: 3.7 milliequivalents-
5 /g of COOH; 0.44 milliequivalents/g free N-terminal NH₂; and 3.48 milliequivalents/g total free NH₂. The molecular weight of the soybean protein ranged from 100 to 2000 D.

b. Modification of soybean protein

The dried soybean protein of step (a) (600 g, 2.5
10 equivalents of total free NH₂) was dissolved in 3L of aqueous 2N potassium hydroxide solution (2.25 mole excess) and the solution was heated at 60°C for 30 minutes. Thereafter, benzenesulfonyl chloride (460 g, 2.60 moles) was added dropwise to the mixture and the reaction temperature was monitored so that it did not
15 exceed 65°C. The reaction continued, with stirring, for 4 hours at 63°C. The reaction mixture cooled to room temperature, then acidified to pH 3.0 with 20% aqueous HCl solution and modified soybean protein precipitated out. The modified soybean protein was then washed twice with distilled water (1L) and dissolved in
20 2N aqueous sodium hydroxide solution until a pH of 8.5-9 resulted. The solution was filtered to remove particulates and the filtrate was reduced and dried in vacuo to give dry modified product (257g, yield = 24%). The product had the following titratable groups: 2.3 milliequivalents/g of COOH; 0.2
25 milliequivalents/g N-terminal free NH₂; and 0.3 milliequivalents- /g total free NH₂.

Example 2: **Modification of soybean protein with benzoyl chloride**

30

A commercial hydrolyzed water solution of soybean protein (AJI-EKI, Ajinomoto USA, Inc.) was used in this Example without further extraction. The protein in solution contained the following functional groups: 2.6 milliequivalents/ml of COOH
35 and 2.0 milliequivalents/ml NH₂. The molecular weight of the

soybean protein was approximately 6.5 kD.

To the soybean solution (240 mL, 0.5 equivalents free NH_2) was added 107 mL of 10N aqueous potassium hydroxide solution followed by 200 mL of distilled water. The solution was then placed in an ice bath (5°C) and benzoyl chloride (70 g, 0.5 moles) was added dropwise within a temperature range between 10 to 25°C . The reaction mixture was then stirred for 4.5 hours at room temperature. The pH of the reaction mixture was then reduced from 13.2 to 2.8 with concentrated HCl. After being allowed to settle for 1 hour, the precipitated modified soybean protein was collected by filtration and washed with distilled water. The soybean protein was then dissolved in 2N aqueous sodium hydroxide solution to give a solution (pH 12.6) which was then evaporated to afford 48 g of dried product (% yield = 41%).

Example 3: Preparation of Empty Microspheres With Modified Soybean Protein

This Example illustrates a method for the preparation and cleaning of empty modified soybean protein microspheres.

PROCEDURE

1. Reagents:

- a. Modified protein powder prepared as described in Example 1
- b. Anhydrous citric acid (USP)
- c. Gum acacia NF
- d. Deionized water
- e. Glacial acetic acid

2. Equipment

- a. pH meter
- b. Eppendorf pipette (0-100ul) and tips
- c. Water bath, 40°C
- d. liquid nitrogen
- e. lyophilization flasks

3. Preparation of Solutions:

- 5 a. Protein solution - Dissolve 100mg modified soybean protein in 1ml deionized water (or multiples thereof). Filter through a Whatman #1 filter paper (if necessary) and keep at 40°C in a water bath. This is solution A.
- b. 1.7 N citric acid with 0.5% acacia - Dissolve 5g of acacia and 109g of citric acid in 1 liter deionized water. Incubate at 40°C. This is solution B.

10 4. Preparation of Microspheres:

- a. Add all of solution A to solution B rapidly in one step while swirling solution B by hand, in a 40°C water bath.

15 Example 4: Preparation of Soybean protein Microsphere containing Encapsulated Insulin

This Example describes a method for the preparation and cleaning of insulin microspheres.

20

PROCEDURE

1. Reagents:

- 25 a. Modified protein powder prepared as described in Example 1
- b. Heparin
- c. Anhydrous citric acid (USP)
- d. Gum acacia NF
- e. Deionized water
- f. Desiccant
- 30 g. Liquid nitrogen

2. Equipment:

- a. Magnetic stirrer
- b. Buret
- 35 c. Microscope
- d. Clinical centrifuge

- e. Dialysis membrane tubing (Spectrum 6, 10 mm, 50,000 M.W. Cutoff)
- f. pH meter
- g. Lyophilizer (Labconco #75035)
- 5 h. Lyophilizing flasks (150-300 mL)
- i. Rotating shell freezer
- j. Isopropanol/dry ice bath or liquid N₂
- k. Mortar and pestle
- l. Storage containers (500 mL)
- 10 m. Eppendorf pipet (0-100 uL)
- n. Plastic closures for dialysis tubing (Spectrum)
- o. 2 mL syringe with 0.45 μ m Acrodisk

3. Preparation of Solutions:

- 15 a. Protein Solution A* (80 mg/ml):
Add 160 mg of modified soybean protein and dissolve to 1 ml with deionized water. Using a 2 ml syringe filter through a 0.45 μ m Acrodisk into a 10 ml test tube. Keep at 40° C.
- 20 b. Solution B (1.7 N citric acid with 1% gum):
Dissolve 10 g of acacia and 109 g of citric acid in 1 liter deionized water.
- c. Solution C (Heparin solution):
Dissolve heparin in Solution B at 150 mg/mL and keep at
25 40° C.

4. Preparation of Microspheres:

- a. Add all of solution A to solution C quickly while swirling solution C slowly, by hand, in a 40°C water
30 bath.

5. Cleaning of Microspheres:

* or multiples thereof.

- a. Transfer the suspension with a syringe (no needle) to dialysis tubing and seal with plastic closures. Tubing should be no more than 70% full.
- b. Discard any amorphous material sedimented and/or aggregated on the surface.
- c. Dialyze the microsphere suspension against acetic acid (using 20 mL of acetic acid solution per ml of microsphere suspension) while stirring the acetic acid solution with a magnetic stirrer.
- d. Replace the acetic acid solution every hour. Continue dialyzing for a total of 3 hours.

6. Lyophilization:

- a. Add one part of 50% Trehalose (Sigma) into nine parts of dialyzed microsphere solution. Flash freeze microspheres in a freeze-drying flask using the shell freezer adjusted to rotate at ca. 190 rpm and immersed in a liquid nitrogen bath.
- b. Freeze dry for 24 hours or until dry as evidenced by lack of self-cooling.
- c. Record weight of dry microspheres.
- d. Grind to a fine powder with mortar and pestle.
- e. Transfer to amber container, seal with desiccant, and store at room temperature.

7. Resuspension:

- a. Weigh the lyophilized powder and calculate the amount of protein in the powder.
- b. Add 0.85 N citric acid into the lyophilized powder at 40°C. The final concentration of protein is 80 mg/ml.

Example 5: Evaluation of Insulin Microspheres in Rats

In this Example, the insulin microspheres prepared in accordance with Example 3 were evaluated in rats. Twelve rats

were divided into two groups as follows:

1. oral insulin microspheres: 3 mg insulin/kg body weight by oral gavage (six rats);
2. Raw insulin (no encapsulation): 3 mg insulin/kg body weight by oral gavage (six rats).

Oral gavage dosing of rats was performed. Insulin microspheres were prepared immediately prior to dosing and Group 1 rats each receive an appropriate dosage of the microsphere suspension. Group 2 rats received the unencapsulated insulin. Approximately 0.5 ml of blood was withdrawn from each rat just prior to dosing ("0" time) and 1 to 6 h post-dosing. Serum from the blood samples were stored at -20°C.

The glucose levels of thawed serum taken from the rats were analyzed by conventional methods. As shown in Figure 1, sharp decreases in serum glucose levels were observed in groups 1 rats receiving the encapsulated insulin. In contrast, the serum glucose levels in group 2 rats slightly increased from t = 0. The results show that encapsulated insulin had a greater biological effect, when administered orally, in contrast to unencapsulated insulin.

Example 6: Preparation of Microsphere Encapsulated Calcitonin

Encapsulation of salmon calcitonin in soybean protein microspheres were performed in the same manner described in Example 3. Calcitonin was obtained from Sandoz (Basil, Switzerland) and a 150 mg/mL calcitonin solution in 1.7 N citric acid solution with 1% gum was prepared as described in Example 3.

Example 7: Evaluation of Calcitonin Microspheres in Rats

In this Example, the calcitonin microspheres prepared in accordance with Example 5 were evaluated in rats. Twelve rats were divided into two groups as follows:

1. oral calcitonin microspheres: 60 µg calcitonin/kg body weight by oral gavage (six rats).

2. oral unencapsulated microspheres: 60 μ g calcitonin/kg body weight by oral gavage (3 rats) (Control).

Oral gavage dosing of rats was performed. Calcitonin microspheres were prepared immediately prior to dosing and Group 1 rats received an appropriate dosage of the microsphere suspension. Group 2 rats received the unencapsulated calcitonin. Approximately 0.5 ml of blood was withdrawn from each rat just prior to dosing ("0" time) and 1 h, 2 h and 3 h post-dosing. Serum from the blood samples were stored at -20°C.

The calcium levels of thawed serum taken from group 1 and 2 rats were analyzed by conventional methods. As shown in Figure 2, sharp decreases in serum calcium levels were observed in group 1 rats receiving the encapsulated calcitonin. In contrast, the calcium levels in group 2 rats slightly decreased from $t = 0$. The results show that encapsulated calcitonin had a greater biological effect, when administered orally, in contrast to unencapsulated calcitonin.

WHAT IS CLAIMED IS:

1 1. A microsphere for encapsulating a cargo, said
2 microsphere comprising a hydrolyzed vegetable protein modified
3 with an amine reactive modifying agent.

1 2. The microsphere according to claim 1, wherein said
2 hydrolyzed vegetable protein comprises acid hydrolyzed soybean
3 protein.

1 3. The microsphere according to claim 1, wherein said
2 microsphere has a diameter equal to or less than 10 microns.

1 4. The microsphere according to claim 1, wherein said
2 amine reactive modifying agent is selected from the group
3 consisting of benzene sulfonyl chloride and benzoyl chloride.

1 5. The composition comprising a biologically-active
2 agent encapsulated within a hydrolyzed vegetable protein
3 microsphere, said microsphere comprising a biocompatible protein
4 wall comprising a hydrolyzed vegetable protein modified with a
5 amine reactive modifying agent.

1 6. The composition according to claim 5, wherein said
2 amine reactive modifying agent is selected from the group
3 consisting of benzene sulfonyl chloride and benzoyl chloride.

1 7. The composition according to claim 5, wherein said
2 biologically-active agent comprises a monoclonal antibody,
3 insulin, calcitonin, or erythropoietin.

1 8. The composition according to claim 5, wherein said
2 hydrolyzed vegetable protein comprises acid hydrolyzed soybean
3 protein.

1 9. A method for preparing a modified hydrolyzed
2 vegetable protein microsphere comprising the steps of:

3 (a) mixing a hydrolyzed vegetable protein with
4 an aqueous alkaline solution to form a solution;

5 (b) treating said solution with an amine reactive
6 agent to form a mixture containing a modified hydrolyzed
7 vegetable protein;

8 (c) recovering said modified hydrolyzed vegetable
9 protein;

10 (d) incubating said modified hydrolyzed vegetable
11 protein in a aqueous acid solution to form said modified
12 hydrolyzed vegetable protein microspheres; and

13 (e) collecting said protein microspheres.

1 10. The method according to claim 9, wherein said
2 aqueous alkaline solution comprises potassium or sodium hydrox-
3 ide.

1 11. The method according to claim 9, wherein step (a)
2 is performed at a temperature ranging between about 35°C and
3 about 40°C.

1 12. The method according to claim 9, wherein said
2 amine reactive agent is selected from the group consisting of
3 benzene sulfonyl chloride and benzoyl chloride.

1 13. The method according to claim 12, wherein said
2 aqueous acid solution further comprises a biologically-active
3 agent.

1 14. The method according to claim 13, wherein said
2 biologically-active agent comprises a monoclonal antibody,
3 insulin, calcitonin, or erythropoietin.

1 15. The microsphere according to claim 1, wherein said

2 hydrolyzed vegetable protein comprises acid hydrolyzed soybean
3 protein.

1 16. The composition according to claim 5, wherein said
2 biologically-active agent is selected from the group consisting
3 of a peptide, a mucopolysaccharide, a carbohydrate, a lipid, a
4 pesticide or any combination thereof.

1 17. The composition according to claim 5, wherein said
2 biologically-active agent is selected from the group consisting
3 of human growth hormone, bovine growth hormone, growth hormone-
4 releasing hormone, an interferon, interleukin-II, insulin,
5 heparin, calcitonin, erythropoietin, atrial naturetic factor, an
6 antigen, a monoclonal antibody, somatostatin,
7 adrenocorticotropin, gonadotropin releasing hormone, oxytocin,
8 vasopressin, cromolyn sodium, vancomycin, desferrioxamine (DFO),
9 or any combination thereof.

1 18. The composition according to claim 17, wherein said
2 biologically-active agent comprises an interferon, interleukin-
3 II, insulin, heparin, calcitonin, oxytocin, vasopressin, cromolyn
4 sodium, vancomycin, DFO or any combination thereof.

19. The composition according to claim 18, wherein said
biologically-active agent is calcitonin.

1 20. A dosage unit form comprising
2 (A) a composition according to claim 5; and
3 (B) (a) an excipient,
4 (b) a diluent,
5 (c) a disintegrant,
6 (d) a lubricant,
7 (e) a plasticizer,
8 (f) a colorant,
9 (g) a dosing vehicle, or

10 (h) any combination thereof.

1 21. A dosage unit form according to claim 20 comprising a
2 tablet, a capsule, or a liquid.

1 22. A method for administering a biologically-active agent
2 to an animal in need of said agent, said method comprising
3 administering orally to said animal a composition as defined in
4 claim 5.

5 23. The composition according to claim 5, wherein said
6 peptide is insulin.

7 24. The composition according to claim 5, wherein said
8 peptide is human growth factor.

9 25. The composition according to claim 5, wherein said
10 peptide is bovine growth factor.

11 26. The composition according to claim 5, wherein said
12 peptide is interferon.

13 27. The composition according to claim 5, wherein said
14 peptide is interleukin II.

15 28. The composition according to claim 5, wherein said
16 peptide is atrial naturetic factor.

17 29. The compositon according to claim 5, wherein said
18 peptide is calcitonin.

19 30. The composition according to claim 5, wherein said
20 biologically active agent is an antimicrobial agent.

21 31. The composition according to claim 5, wherein said

22 biologically active agent is a quinolone.

23 32. The composition according to claim 5, wherein the
24 biologically active agent is a polysaccharide.

25 33. The composition according to claim 32, wherein said
26 polysaccharide is heparin.

27 34. The composition according to claim 5, wherein said
28 biologically active agent is an antigen.

29 35. The composition according to claim 5, wherein said
30 biologically active agent is a monoclonal antibody.

31 36. A composition according to claim 5, wherein said
32 biologically active agent is aspirin.

33 37. The method according to claim 5, wherein said
34 microspheres are hollow and said biologically-active agent is
35 encapsulated therein.

1/4

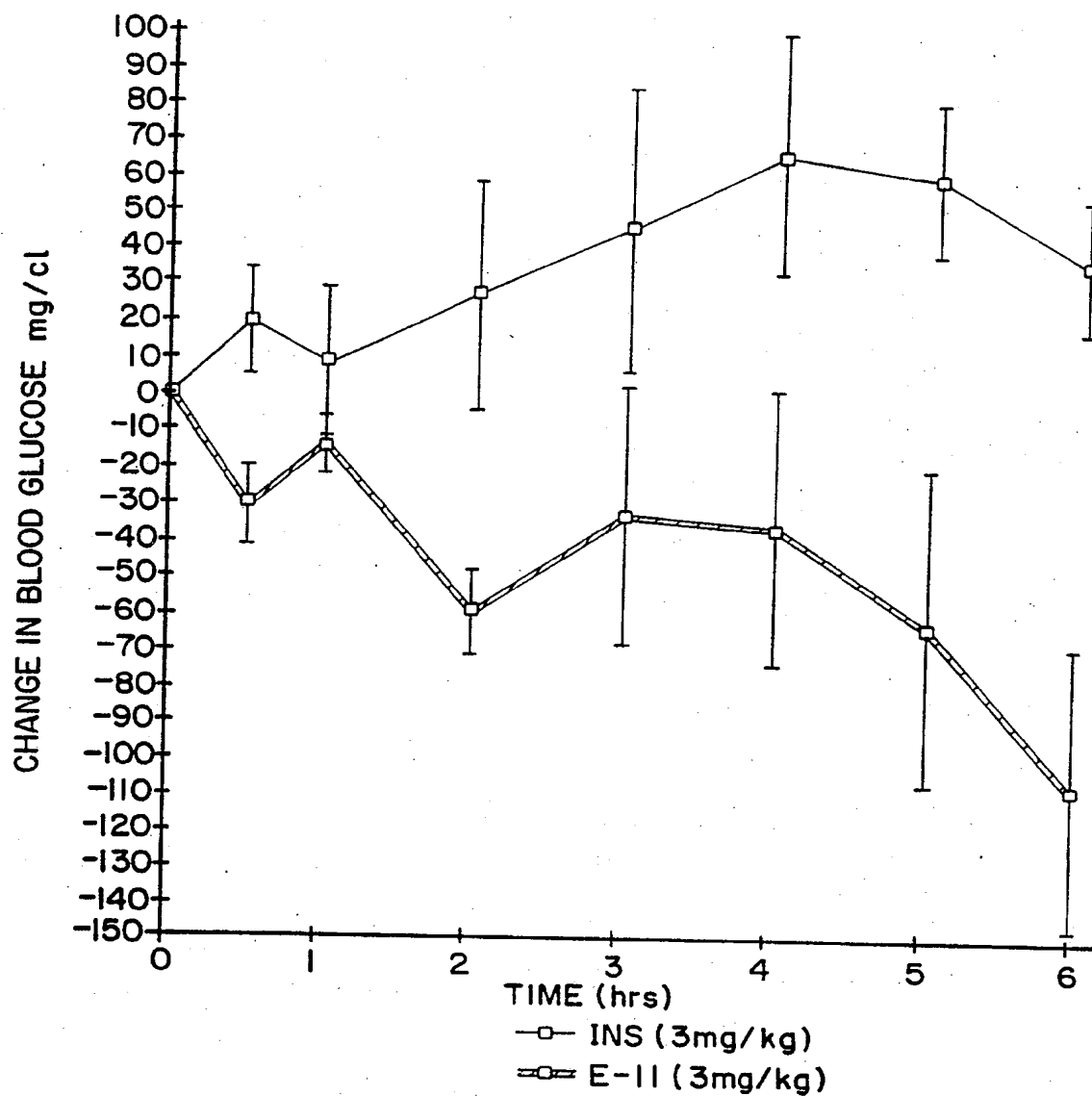


FIG. 1

2/4

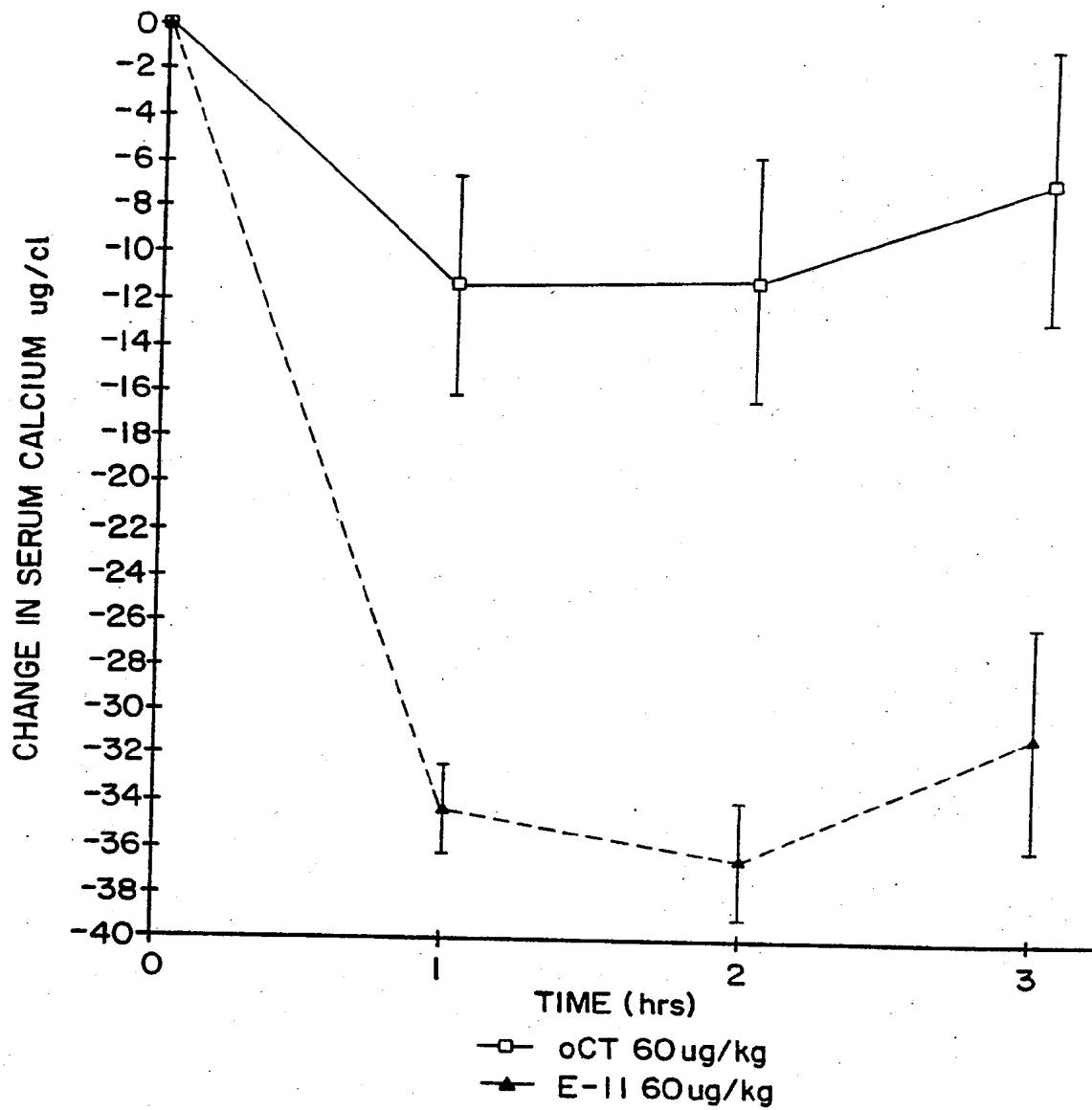


FIG. 2

3/4

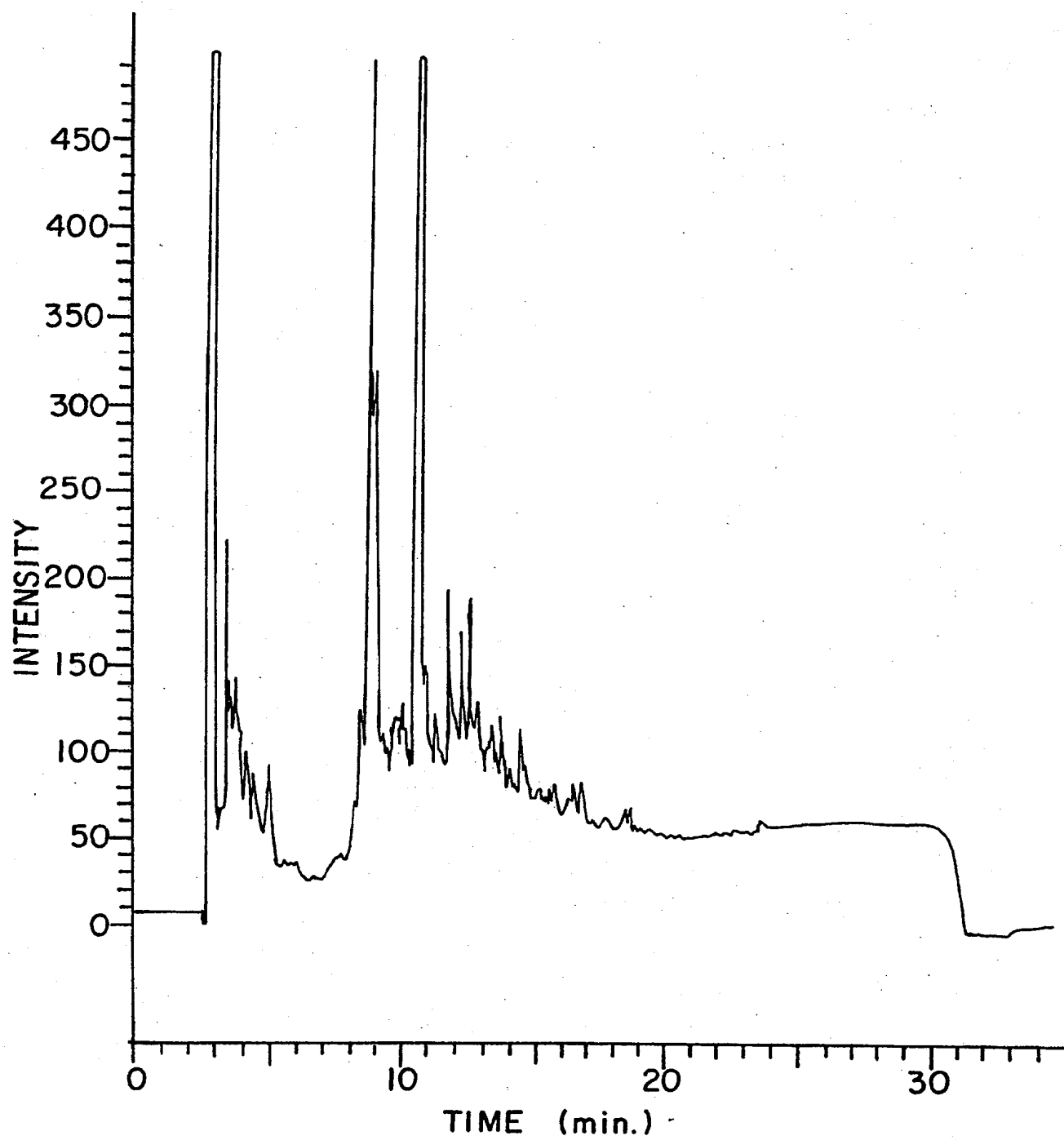


FIG. 3

SUBSTITUTE SHEET (RULE 26)

4/4

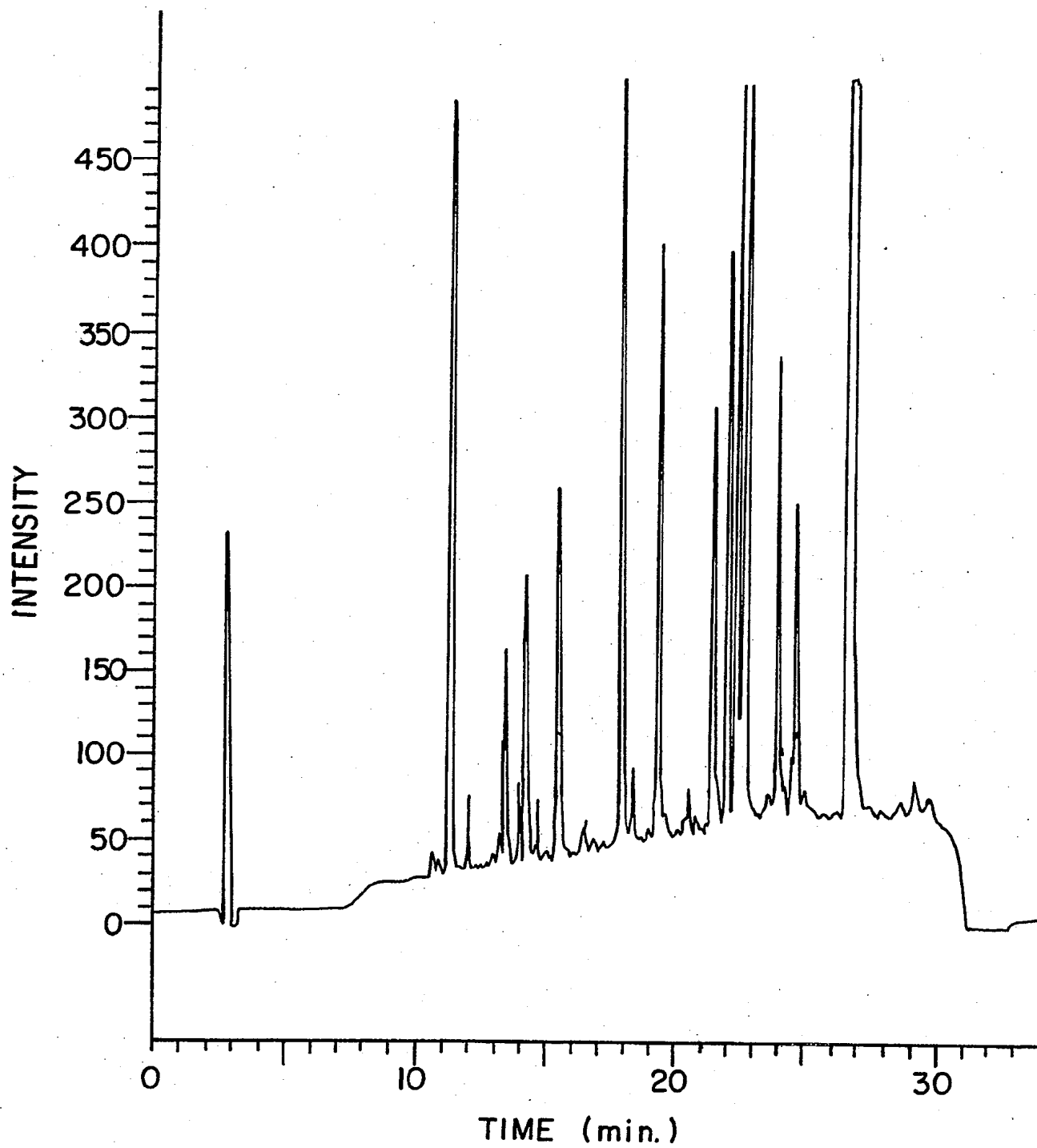


FIG. 4

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04561

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 9/16

US CL :424/491, 498, 489

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/491, 498, 489, 426

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 3,565,559 (SATO) 23 February 1971, see entire document.	1-37
Y	US, A, 5,100,669 (HYON) 31 March 1992, see entire document.	1-37

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

"A"	document defining the general state of the art which is not considered to be part of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

08 JULY 1994

Date of mailing of the international search report

10 AUG 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

THURMAN K. PAGE

Telephone No. (703) 308-2351

Form PCT/ISA/210 (second sheet)(July 1992)*

